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Increased Expression of the Stefin A Cysteine Proteinase Inhibitor Occurs in the Myelomonocytic Cell-Infiltrated Tissues of Autoimmune Motheaten Mice

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Mice homozygous for the motheaten (me) and viable motheaten (me) mutations develop a progressively severe inflammatory disease in association with profound disruption of normal hematopoiesis. These mutant mice have been previously shown to manifest abnormally high expression in the bone marrow of cDNAs encoding three members of the stefin family of cysteine proteinase inhibitors. The data reported here reveal that increases in levels of both stefin transcripts and proteins occur in bone marrow, splenic and pulmonary tissues of me and me^v mice, and correlate with the abnormal expansion of stefin A-producing myelomonocytic cells in these tissues. Increases in stefin expression are also apparent in me and me^v skin and appear to reflect focal hyperplasia of stefin-producing epidermal cells as well as infiltration by stefin-expressing monocytic and granulocytic cells. Because the increases in stefin protein levels occur in the same tissues that are most adversely affected by the me mutation, it appears that overexpression of these proteins may distinguish the cell population responsible for disease pathogenesis and may be relevant to the severe tissue inflammation and damage observed in these mutant mice. (Am J Pathol 1994, 145:902–912)

Mice homozygous for the autosomal recessive motheaten (*me*) and the less severe allelic viable motheaten (*me^v*) mutations are severely immunodeficient and develop systemic autoimmune disease early in life.[1–3] Affected animals are distinguished by the patchy absence of pigment and alopecia, runting, the development of progressively severe glomerulonephritis, arthritis with limb necrosis, and hemorrhagic pneumonitis, which causes death by age 3 (*me*) or 6 (*me^v*) weeks. These defects arise consequent to severe disruption of normal hematopoietic cell development and function. Lymphoid ontogeny is markedly abnormal, with the mutant mice displaying a paucity of bone marrow B220$^+$ B cell progenitors and early thymic involution with subsequent reduction in T cell number and function.[4,5] Similarly, both differentiation and activity of natural killer cells are impaired in these mice.[7,8] Despite the reduction in normal lymphopoiesis, *me* and *me^v* mice produce high titers of autoreactive antibodies, an abnormality associated with overexpansion in the periphery of CD5$^+$ B cells, a putative autoreactive subset normally constituting a very minor portion of the peripheral B cell population.[6]

Monocyte/macrophage and neutrophil numbers are also enormously increased and the accumulation of these latter cell types in tissues such as the skin and lung represents the major factor responsible for the pathology. Because all of these abnormalities can be transferred into irradiated normal mice by *me* or *me^v* bone marrow, the abnormality underlying the *me* and *me^v* phenotype has long been ascribed to a primary hematopoietic cell defect.[9,10] This contention has now been verified by the recent data from our group

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linking the *me* phenotype to loss of function mutation in the gene encoding a tyrosine phosphatase, PTP1C, which is predominantly expressed in hemopoietic cells.[11,12] However, at present the precise biological functions of PTP1C are unknown and the cellular and biochemical events whereby impaired expression of this phosphatase causes the multiple phenotypic abnormalities seen in *me* mice remain to be determined.

To begin elucidating the pathophysiology of the severe inflammatory disease expressed in *me* mice, we undertook studies aimed at delineating some of the biochemical abnormalities that occur in *me* and *me*^{v} hemopoietic cells, presumably as secondary consequences to loss of PTP1C function. To this end, a differential screening strategy was used to identify genes that are uniquely or preferentially expressed in *me*^{v} relative to normal hemopoietic cells. These studies led to the identification of three cDNAs that were overexpressed in *me*^{v} compared with normal bone marrow cells.[13] Based on the deduced amino acid sequences, these cDNA encode three closely related proteins of molecular weights 12.7, 13.8, and 14.1 kd, which belong to the stefin family of cysteine proteinase inhibitors (CPI) and were thus designated as Stefin (Stf)-1, -2, and -3, respectively.[13] The CPIs represent a large superfamily of highly conserved proteins that act as potent reversible and competitive inhibitors of endopeptidases of the papain superfamily and of lysosomal cathepsins, such as cathepsin B, H, and L.[14,15] Structural and functional differences between the members of this family have led to their assignment into three subfamilies; the stefins, the cystatins, and the kininogens.[14–16] Depending on their biochemical properties and tissue distribution, members of the stefin family are in turn further subclassified into A (acidic) and B (neutral) subtypes[14] and based on these considerations the Stf-1, -2, and -3 cDNAs isolated from *me*^{v} bone marrow appear to encode stefin A proteins. Thus, it appears that *me* bone marrow contains one or more cell populations characterized by abnormally high expression of three members of the stefin A family of CPIs.

Although CPIs are recognized as the major endogenous inhibitors of lysosomal cysteine proteinases, little information is available concerning the relevance of CPIs to specific aspects of cell behavior. However, altered expression of both cysteine proteinases and their inhibitors has been described in conjunction with a broad spectrum of disease, most notably systemic autoimmune, inflammatory, and neoplastic states.[19–21] Although the importance of these proteins to tissue damage and dysfunction in these disorders is not clear, the finding that stefin gene expression is increased in *me* and *me*^{v} compared with normal bone marrow cells suggests that these CPIs are of pathogenetic relevance. To address this possibility, we have now conducted more extensive analysis of stefin expression in *me* and *me*^{v} mice. As reported here, the results of these studies indicate that overexpression of stefin occurs predominantly in myelomonocytic cell lineages and suggest that the apparent increases in stefin levels in various *me* tissues reflects abnormal expansion of stefin-producing macrophage/myeloid populations in these tissues. The data also indicate that increases in stefin protein occur primarily in the tissues most adversely affected by the *me* and *me*^{v} mutations and therefore demarcate the presence of cell populations that are likely to play a critical role in the pathogenesis of the autoimmune/inflammatory disease observed in these mutant mice.

Materials and Methods

Mice

Original breeding pairs for C57 BL/6J *me*^{v}/+ and +/+ and C3HeBFeJ *me*/+ and +/+ were obtained from the Jackson Laboratory (Bar Harbor, ME) and were bred in the animal facility at the Samuel Lunenfeld Research Institute, Mount Sinai Hospital. All mice used in these studies were 9 to 14 days old.

Antibodies

The following monoclonal antibodies were prepared in the laboratory from hybridoma culture supernatant: anti-Thy1.2, anti-B220, anti-Mac-1, and anti-Gr-1 antibodies produced by the J1j.10(ATCCTIB184), RA3–3A1/6.1(ATCCTIB146), M1/70.15.11.5HL(ATCCTIB128), and RB6–8C5 hybridoma clones, respectively. Hybridomas were grown in Dulbecco's minimum essential medium (GIBCO BRL, Gaithersburg, MD) with 10% heat-denatured fetal calf serum (FCS; Bioproduct Inc., Burlingame, CA) and 10^{-5} M β-mercaptoethanol (GIBCO BRL) at 37 C with 5% CO_2. Supernatants containing monoclonal antibodies were harvested after centrifugation and used directly without concentrating. Antibodies were detected using fluorescein isothiocyanate (FITC) conjugated goat anti-rat Ig $F(ab')_2$ fragments (Tago Inc., Burlingame, CA). Phycoerythrin (PE)-conjugated rat anti-mouse Gr-1 (Immunochemicals; Southern Biotechnology Inc., Birmingham, AL) was also used for two-color fluorocytometric analyses. Rabbit anti-rat stefin A was a kind gift of Dr. W. Epstein[22] (UCSF, San Francisco, CA). Immunohistochemistry was conducted using biotinylated goat

anti-rabbit IgG (Vector Dimension Labs, Burlingame, CA) and horseradish peroxidase-conjugated streptavidin (Vector Dimension Labs). PE-conjugated goat anti-rabbit IgM and IgG (Immunochemicals) was used for immunofluorescence staining.

RNA Extraction and Northern Blotting

Total RNA was extracted from tissues of wild-type and mutant mice as previously described.[23] Briefly, tissues were quick-frozen on dry ice, then homogenized in guanidine isothiocyanate and after acid phenol extraction RNA was precipitated with isopropanol. For Northern analysis, 20-μg RNA samples were subjected to electrophoresis on 1% agarose-formaldehyde gel in a 1X MOPS running buffer. The RNA was then transferred to Gene Screen nylon membranes (New England Nuclear, Boston, MA), immobilized by ultraviolet cross-linking (Stratagene crosslinker), and hybridized at 65 C in phosphate buffer with the 0.4-kb $^{32}P\alpha$CTP-labeled murine stefin-1 cDNA.[13] After hybridization, membranes were washed in 150 mmol/L phosphate buffer containing 0.1% sodium dodecyl sulfate (SDS) at 60 C until the background was lower than 200 cpm. Membranes were exposed to X-ray film (X-omat; Kodak, Rochester, NY) with intensifying screens at −70 C. To control for loading differences, blots were stripped in 0.1% SDS for 10 minutes at 95 C and reprobed with a 300-bp murine β-actin gene fragment.

Protein Extraction and Western Blot Analysis

Total cellular protein lysates were prepared by resuspending freshly isolated bone marrow, spleen, or thymic tissues in 1 ml of lysis buffer (2.5 mmol/L Tris, pH 8.3, 150 mmol/L $ZnCl_2$, 50 μmol/L NaF, 50 μmol/L Na_2HPO_4, 2 μmol/L EDTA, 1% NP-40, 2 mmol/L PMSF, 2 mmol/L sodium vanadate). Lysates were centrifuged for 20 minutes at 10,000 × *g* and 40 μg of total cell lysate protein was subjected to electrophoresis under reducing conditions on 15% SDS-polyacrylamide gels. The proteins were electroblotted onto nitrocellulose paper (Schleicher & Schuller Inc., Keene, NH) and membranes were then blocked with 5% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) in Tris-buffered saline (TBS) and incubated overnight with anti-stefin A antibody (25 μg/ml) in 5 ml of blocking solution. The filters were then incubated for 2 hours at room temperature with 1 μCi of ^{125}I-labeled protein A (Amersham Corp., Oakville, Ontario, Canada) in 5 ml of blocking solution, then washed and exposed to Kodak X-omat film at −70 C.

Immunofluorescence Analyses

Cell Suspensions

Single cell suspensions were prepared from spleen by pressing the tissue between the frosted ends of two microscopic slides and from bone marrow by flushing the marrow with 1 cc of medium. Cells in suspension were washed and the red blood cells lysed in hypotonic solution. Viability of the cells was evaluated by staining in 0.4% trypan blue (Sigma Chemical Co.). Cells prepared in this manner (1×10^6) were stained for cell surface markers using rat anti-mouse monoclonal anti-Mac-1, Gr-1, Thy1.2, and B220 antibodies, respectively, as primary antibody and FITC-conjugated rat anti-mouse Ig as secondary antibody. Nonspecific staining was assayed using secondary antibody alone. Expression of the various cell surface antigens was then analyzed on a FACS II analyzer using the LYSES program software (Becton Dickinson, Mountain View, CA). For detection of cytoplasmic protein, cells were permeabilized by incubation with 100% MeOH at −20 C for 10 minutes. The cells were then extensively washed, blocked in 10% goat serum (Vector Labs), and incubated for 30 minutes at 4 C with the primary rabbit anti-stefin A and secondary goat anti-rabbit PE-conjugated antibodies. Nonspecific staining was assessed using secondary antibody alone. After staining, cells were washed in phosphate-buffered saline (PBS) containing 0.1% FCS and 0.01% sodium azide, fixed in 1% paraformaldehyde (Sigma Chemical Co.), and analyzed by flow fluorocytometry or fluorescence microscopy.

Immunohistochemistry

Tissues were harvested from normal and mutant mice, fixed in 4% paraformaldehyde, dehydrated, and paraffin embedded. Five-micron sections were cut, deparaffinized, rehydrated, treated with 0.5 mg/ml protease (type XXIV; Sigma Chemical Co.) and 3% H_2O_2 in MeOH, then blocked with 10% normal goat serum. Sections prepared in this manner were incubated for 2 hours with rabbit antistefin A antibody (25 μm/ml) then for 1 hour with biotinylated goat anti-rabbit IgG secondary antibody (Vector Labs, 1:200) and for 1 more hour with streptavidin-horseradish peroxidase complex (1:400) 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical Co.) was used as a substrate for horseradish peroxidase (0.5

mg/ml DAB in Tris, pH 7.6, with H_2O_2). Each incubation was followed by three washes in PBS. The slides were counterstained with methyl green, dehydrated, cleared, and mounted with De Pex (BDH Ltd., Toronto, Ontario, Canada).

Statistical Analysis

Student's *t*-test (using pooled variance estimate) was used to compare the percentages of each cell type found in *me* or *me*^{v} spleen and bone marrow with those found in wild-type spleen and bone marrow. $P \leq 0.05$ was considered statistically significant.

Results

Stefin Gene Expression in Motheaten Versus Normal Tissues

In previous studies, stefin gene expression was found to be markedly increased in motheaten relative to normal bone marrow[13] but the spectrum of other tissues showing this abnormality was not defined. To fully delineate the spectrum of motheaten tissues showing altered stefin expression, expression of the gene was examined by Northern analysis in both hemopoietic and nonhemopoietic tissues of these mice. As previously reported, stefin expression was found to be negligible in normal mouse bone marrow, whereas a single highly abundant stefin transcript of ≈600 bp was detected in *me*^{v} and *me* bone marrow cells using the Stf-1 (Figure 1). Stf-2, or Stf-3 (data not shown) cDNAs as probes. Relatively high levels of stefin expression were also observed in *me*^{v} (Figure 1) and *me* (data not shown) splenic tissues. By contrast, however, stefin transcripts were not detected in thymic or in most nonhematopoietic tissues (with the exception of lung) from either *me* or normal mice. Therefore, the increased levels of stefin expression detected in *me* bone marrow and splenic tissues appear to arise in non-T cell-related hemopoietic lineages.

To determine whether stefin protein was also relatively increased in abundance in motheaten mice, stefin expression was also examined in bone marrow and splenic tissues from these mice using a polyclonal antibody raised against rat stefin A[22] and shown to be reactive with murine stefin A as well. This analysis revealed the presence in motheaten bone marrow and spleen of a stefin species of about ≈12 to 14 kd (Figure 2), as is consistent with the predicted molecular weights of 12.7, 13.8, and 14.1 kd for Stf-1, -2, and -3, respectively.[13] As shown in Figure 2, levels of stefin protein expression also appeared increased in *me* relative to normal mice, although the differences were less striking than those observed at the level of mRNA. For example, results of densitometric analysis indicated levels of stefin transcripts and proteins to be 100- and 60-fold increased, respectively, in *me* compared with normal bone marrow (data not shown). Whether these differences in the relative increases of transcript compared with protein levels reflect post-transcriptional factors modulating the extent to which stefin transcript is translated to protein or, alternatively, relate to the different assay systems used remains to be determined. However, the finding of increased stefin protein in motheaten hemopoietic cells

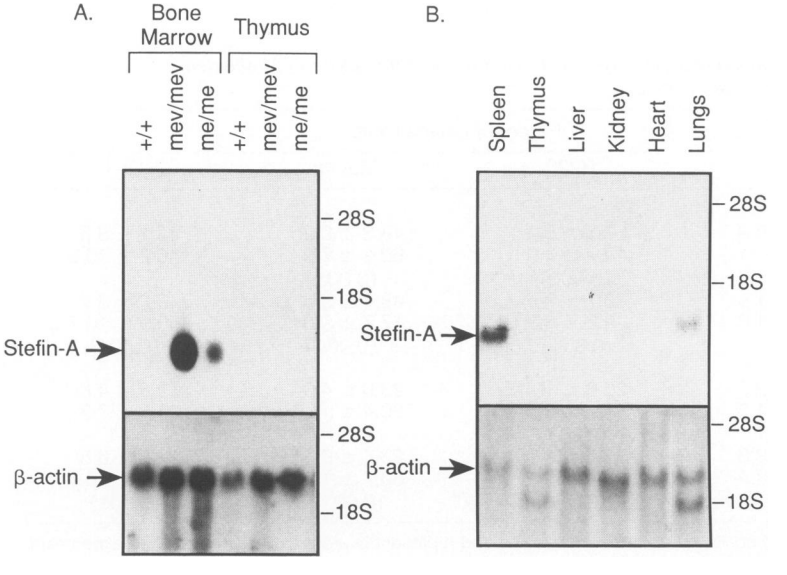

Figure 1. *Northern blot analysis of stefin A expression in* me, me^v, *and wild-type tissues. Total cellular RNA (20 μg) from bone marrow and thymus of C57BL/6J wild-type,* me^v *homozygous, and* me *homozygous mice* (**A**) *and from various tissues of* me^v *homozygous mice* (**B**) *was loaded into each lane, electrophoresed over 1% formaldehyde/agarose, and transferred to nitrocellulose. Filters were probed with the full-length* ^{32}*P-labeled Stf-1 cDNA and then washed and reprobed with a* ^{32}*P-labeled actin gene fragment. Positions of the stefin A and β-actin transcripts are shown on the left.*

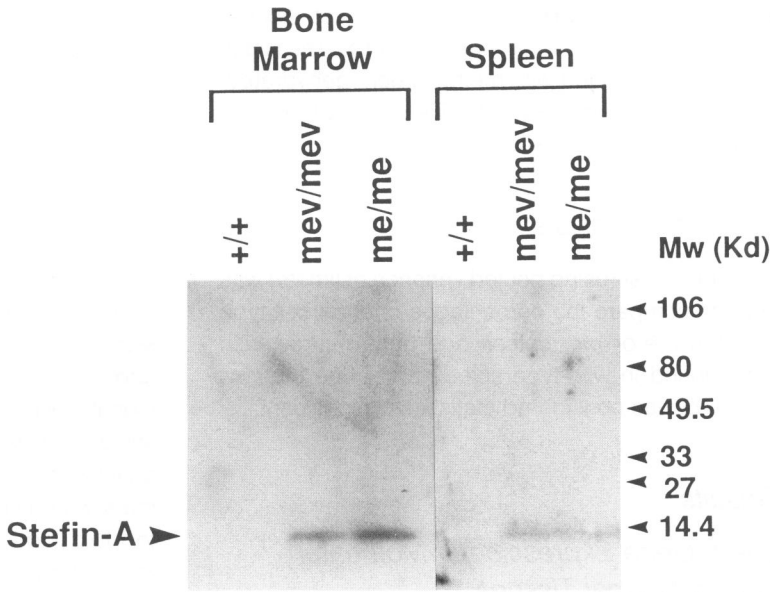

Figure 2. *Immunoblot analysis showing stefin A expression in* me, me^{v}, *and wild-type bone marrow and spleen. Total cell lysates (40 μg) from wild-type,* me^{v}, *and* me *bone marrow or spleen were loaded into each lane, electrophoresed through SDS-PAGE, transferred to nitrocellulose, and immunoblotted with rabbit anti-rat stefin A antibody and* ^{125}I*-labeled protein A. The positions of molecular weight markers are shown on the right and of stefin A species on the left.*

suggests that the increases in stefin gene expression may be relevant to disease pathogenesis in these mice.

Profile of Stefin Gene Expression in Different Hemopoietic Cell Lineages

To determine which cell types in motheaten bone marrow and spleen are responsible for the increases in stefin expression detected in these tissues, stefin expression was next examined in relationship to cell lineage using immunofluorescence analysis. For these studies, antibodies to the cell surface antigens Thy1.2, B220, Mac-1, and Gr-1 were used to identify T cells, B cells, macrophages, and granulocytes, respectively, and rabbit anti-rat stefin A antibody was used to identify cells expressing cytoplasmic stefin A protein. Before investigating the cell profile of stefin expression, the relative proportions of each cell type were first compared between *me* and normal mice. As noted above, data from previous studies of these mutant mice indicated a reduction in numbers of conventional (ie, B220^{+}) B cells and T cells in contrast to increases in the numbers of macrophages and granulocytes. As indicated by the data shown in Table 1, the results of the current analysis confirm the presence of reduced numbers of B cells (B220 positive) and increased numbers of macrophage/monocytes (Mac-1 positive) and granulocytes (Gr-1 positive) in *me* and *me*^{v} relative to wild-type bone marrow and spleen.

Table 1. *Relative Percentages of Various Hemopoietic Cell Types in Bone Marrow and Spleen of Motheaten, Viable Motheaten, and Wild-Type Congenic Mice*

	Percent Positive Cells*			
	Thy1.2	B220	Mac-1	Gr-1
Bone marrow				
C57BL/6J +/+	3.9 ± 0.9	30.5 ± 6.1	48.3 ± 9.2	43.1 ± 8.5
C57BL/6J *me*^{v}/*me*^{v}	3.2 ± 1.1	14.7 ± 4.8 (0.001)†	62.2 ± 7.9 (0.001)	46.2 ± 10.9
C3HeFeJ +/+	4.6 ± 1.5	30.7 ± 7.6	48.7 ± 5.6	40.2 ± 3.7
C3HeFeJ *me*/*me*	3.9 ± 1.8	9.6 ± 5.3 (0.001)	77.7 ± 9.0 (0.001)	59.9 ± 10.7 (0.001)
Spleen				
C57BL/6J +/+	14.9 ± 2.0	28.6 ± 10.1	23.6 ± 4.0	22.6 ± 4.8
C57BL/6J *me*^{v}/*me*^{v}	7.5 ± 4.2 (0.001)	17.7 ± 9.5 (0.01)	30.4 ± 11.5	21.5 ± 7.3
C3HeFeJ +/+	16.1 ± 3.8	35.7 ± 6.2	28.4 ± 6.4	22.0 ± 6.8
C3HeFeJ *me*/*me*	9.8 ± 3.4 (0.001)	15.7 ± 6.4 (0.001)	50.7 ± 13.7 (0.001)	39.9 ± 10.7 (0.001)

* Values are means ± SD of the percentages of positively stained lymphocytes and represent averages of 10 independent experiments.
† Bracketed numbers show *P* values for mutant (*me*^{v}/*me*^{v} or *me*/*me*) *versus* wild type (+/+); only statistically significant values ($P \leq 0.05$) are shown.

Although no differences were detected in the numbers of T cells (Thy1.2 positive) in *me* and wild-type bone marrow, T cell number was diminished by about twofold in *me* relative to wild-type spleen ($P = 0.001$). The results of this analysis also revealed a relative expansion of granulocytic (Gr-1 positive) cells in the *me* but not *me*^{v} hemopoietic tissues (Table 1). It should be noted, however, that a previous analysis of specificity of the antibodies used here revealed that a portion of the Mac-1- and Gr-1-positive cell populations showed dual positivity for these surface antigens and, thus, that these markers do not entirely demarcate single lineage populations. Therefore, the increases in numbers of Mac-1-positive cells apparent in motheaten spleen and bone marrow may reflect expansion of several cell populations, including those of either monocyte/macrophage or myeloid origin.

Immunofluorescence analysis was next used to determine the percentages of cells expressing cytoplasmic stefin A protein in *me* bone marrow and to investigate which cell types accounted for detection of increased stefin expression in these animals. As anticipated, *me* bone marrows showed considerably more stefin A-positive cells (on average ≈15%) than were detected in normal bone marrow (2 to 3%; data not shown). As illustrated in Figure 3, the results of dual color flow cytometric studies in which expression of cytoplasmic stefin A and the lineage-specific surface markers was examined coincidentally revealed stefin A expression in both wild-type and *me* bone marrow to be primarily restricted to the cell populations demarcated by anti-Mac-1 and anti-Gr-1 staining. Thus, in wild-type mice approximately 3% of stefin A-positive cells were also Mac-1 or Gr-1 positive, whereas virtually none of the B220- and Thy1.2-positive cells showed stefin A expression. Similarly, in *me* bone marrow stefin A expression was almost exclusively found in the Mac-1 and Gr-1-positive population; in one animal expression of stefin A was detected in 24 and 15% of the Mac-1- and Gr-1-positive bone marrow cells, respectively (Figure 3). These results therefore indicate that the increases in stefin expression observed in motheaten bone marrow map primarily to a population of myelomonocytic cells and probably reflect the abnormal overexpansion of these cells in the marrow.

Tissue Distribution of Stefin A-Expressing Cells

Previous studies of the pathological abnormalities found in *me* mice have revealed that most of the morbidity associated with this syndrome reflects tissue infiltration by an overexpanded population of macrophages/monocytes and neutrophils.[2,3] Accu-

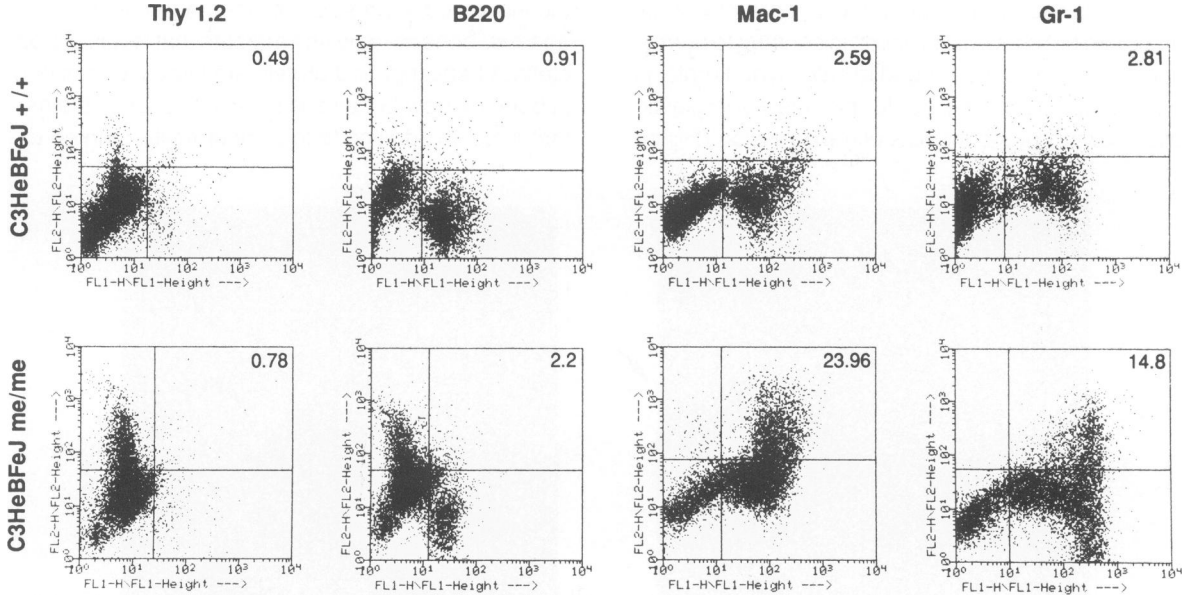

Figure 3. *Representative example of two-color immunofluorescence analysis showing the expression of stefin A in various cell lineages in* me *and wild-type bone marrow cells. The* me *and wild-type control bone marrow cells were stained sequentially with rat anti-mouse Thy1.2, B220, Mac-1, and Gr-1 antibodies followed by FITC-goat anti-rat Ig, rabbit antistefin A antibody, and PE-donkey anti-rabbit Ig. Samples were analyzed by flow cytometry and the data displayed as two-dimensional plots with the ordinate showing FITC-conjugated antibody staining and the abscissa showing PE-conjugated antibody staining. The four quadrants demarcated by the lines within each matrix represent unstained cells (lower left quadrant), cells stained with both fluorochromes (upper right), and cells stained with a single fluorochrome (upper left and lower right). Numbers shown in the upper right quadrants indicate the percentage of double-stained cells.*

mulations of these cells in the skin results in the severe dermatitis that lends these mice their motheaten appearance, whereas in the lung the infiltrating myelomonocytic cells induce intra-alveolar hemorrhage and pneumonitis, which represents the primary cause of death. To determine whether the distribution of stefin A-expressing cells in *me* mice parallels that of these tissue-infiltrating cell populations, the pattern of stefin A expression was assessed in various tissues of these mice by immunohistochemical analysis with the polyclonal antistefin A antibody. Analysis of *me* splenic tissue in this manner revealed the presence of large clusters of stefin-expressing cells that were widely distributed throughout the spleen and associated with loss of the normal splenic architecture (Figure 4). By contrast, far fewer stefin-producing cells were detected in normal spleen and these dispersed cells were apparent only in the red pulp.

In contrast to spleen, pulmonary tissues from normal mice were completely lacking in stefin-expressing cells (data not shown). However, as is consistent with the progressive development of severe inflammatory lesions in *me* lungs, multiple foci of stefin-producing cells were observed throughout both the pulmonary interstitial tissues and alveoli of the mutant animals (Figure 5). Stefin expression was also assessed in *me* skin, and, as shown in Figure 5, was detected in all layers of the epidermis. As is consistent with previous data concerning the tissue distribution of stefin A,[24–26] stefin expression was also detected in the epidermal epithelial cells of normal mice (data not shown). However, in contrast to normal mice, the *me* mice were found to have hyperkeratosis and hyperplasia of the epidermis, the hyperplastic cells showing pronounced stefin expression (Figure 5). The *me* skin was also distinguished from that of normal mice by the presence in the former of infiltrating populations of stefin-producing cells in the dermal connective tissues (Figure 5). In contrast to the abnormalities detected in spleen, lungs, and skin, the tissues that are severely affected by the *me* mutation, *me* and normal mice showed comparable stefin expression in all other tissues studied (data not shown).

These results therefore reveal a correlation between the tissue distribution of stefin-producing cells and myelomonocytic inflammatory lesions in *me* mice and thus provide further evidence linking increases in stefin expression in various *me* and *me*^{v} tissues with the expansion in these tissues of Mac-1- and Gr-1 positive hemopoietic cell populations. The results also suggest that in at least some tissues of the mutant mice increases in stefin expression reflect increases in the numbers of not only hemopoietic but also epithelial-derived cells.

Discussion

Previous investigations into the pathogenesis of the inflammatory syndrome manifested by *me* and *me*^{v} mice led to the identification of three members of the CPI stefin gene family, which appeared to be markedly overexpressed in motheaten compared with normal bone marrow.[13] The data reported here confirm the increased expression of stefin A transcript in *me* and *me*^{v} bone marrow and indicate that levels of both stefin A transcript and protein are increased not only in bone marrow but also in splenic tissues of the mutant mice. In addition to this abnormality, marked ex-

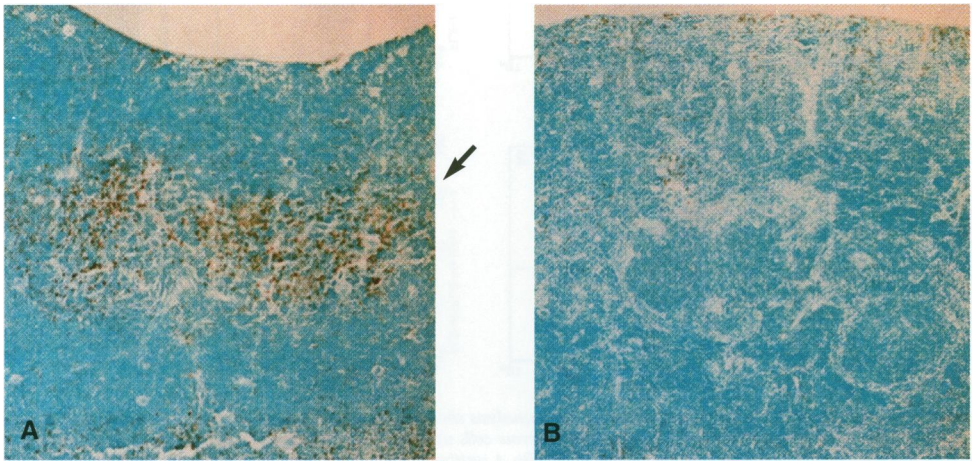

Figure 4. *Immunohistochemical analysis showing stefin A-expressing cells in* me *and wild-type spleen. Stefin A expression was detected in* me *(left panel) and wild-type (right panel) splenic tissue by staining with rabbit anti-rat stefin A and biotinylated goat anti-rabbit Ig antibodies and horseradish peroxidase-conjugated streptavidin and DAB as described in Materials and Methods. Tissues were then counterstained with methyl green and examined by light microscopy (magnification × 100). The arrow indicates a focus of stefin-producing cells (brown staining) in the* me *spleen.*

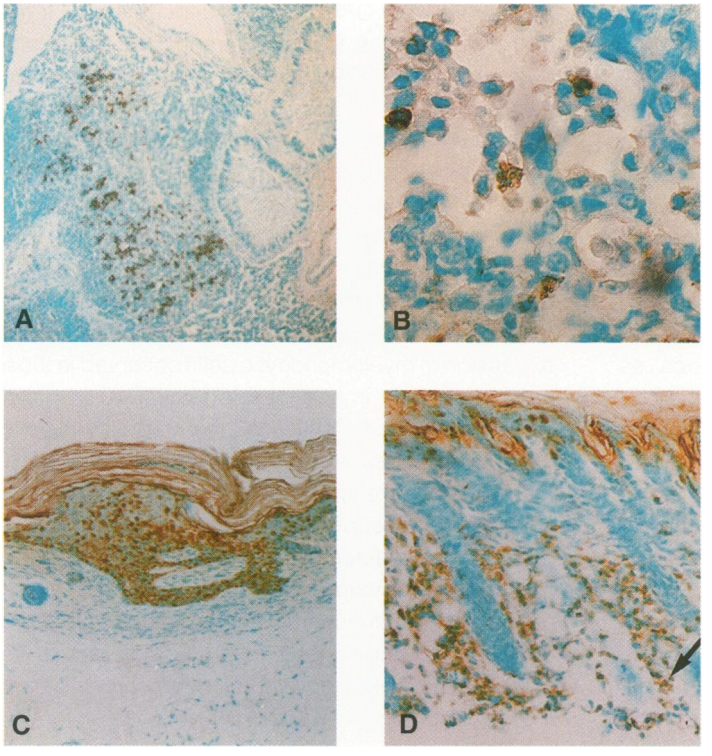

Figure 5. *Immunohistochemical analysis showing stefin A-expressing cells in* me *lung and skin. Stefin A expression was detected in* me *lung (upper panels) and skin (lower panels) by staining with rabbit antistefin A and biotinylated goat anti-rabbit Ig antibodies and horseradish peroxidase-conjugated streptavidin and DAB. Tissues were then counterstained with methyl green and examined by light microscopy (magnification ×100, panels on the left; ×1000, panels on the right). Accumulations of stefin-expressing cells (brown staining) are present in both the pulmonary interstitium and alveoli (upper panels) and in all layers of the epidermis, including the hyperplastic and hyperkeratotic areas (lower panels) in* me *mice. The arrow indicates perifollicular stefin-expressing cells in the dermal connective tissue of* me *skin.*

pansion of myelomonocytic cell populations also distinguished the bone marrow and splenic tissue of motheaten mice from that of normal animals. Similarly, stefin A expression was also abnormally prominent in the skin and lungs of *me* mice, tissues in which massive monocyte/granulocyte accumulation occurs and is considered responsible for organ damage and dysfunction. These observations indicate that overexpression of stefin A proteins in *me* and me^{v} mice occurs for the most part in the same tissues that are most adversely affected by the mutation and suggest that this abnormality reflects the expansion of stefin-producing myelomonocytic cell populations within these tissues.

Based on data from previous studies addressing the normal tissue distribution of stefin proteins, stefin appears to be expressed most prominently in epithelial cells, neutrophil granulocytes, and activated monocyte/macrophage cells.[22,24,25] Because these latter cell types also represent the lineages known to be massively expanded in motheaten mice,[2,3] it appeared that the increases in stefin levels observed in motheaten tissues might reflect expansion in the numbers of stefin A-producing cells rather than increased expression of the protein in individual cells. This issue was addressed by comparing the numbers of stefin-expressing cells and monocyte/macrophage and myeloid cells in motheaten and normal bone marrow. The data indicate that the numbers of both myelomonocytic cells and stefin-expressing cells are markedly increased in *me* compared with normal bone marrow and also reveal stefin A expression in bone marrow to be largely restricted to the Mac-1-and Gr-1-positive cell populations. These data therefore support the contention that increases in stefin levels in *me* and *me*^{v} tissues reflect the presence of abnormally high numbers of stefin-expressing myelomonocytic cell populations within these tissues. Interestingly, the percentages of motheaten bone marrow cells co-expressing stefin A and either the Mac-1 or Gr-1 antigens (24 and 15%, respectively) were considerably lower than the percentages of Mac-1 and Gr-1 cells in the bone marrow (78 and 60%, respectively). This finding may relate to the possibility that the cell populations distinguished by expression of Mac-1 and/or Gr-1 are heterogeneous and contain cells that differ with respect to their states of activation and/or differentiation and, hence their capacity for stefin A expression. Along similar lines, although the association of the *me* phenotype with the expansion of a hyperproliferative, growth factor-independent population of myelomonocytic cells is well established,[27,28] it is unclear whether this population is comprised of a single or multiple cell types. Further studies of stefin A expression in *me* mice may provide a means to address this issue and thereby increase our understanding of the differentiation pathways involved in the genesis of myeloid and monocytic cells.

Although the biochemical and enzymatic properties of stefins have been extensively characterized,[26] the relevance of these CPIs to specific facets of cell behavior remain unknown. However, cysteine proteinases that represent the substrates for CPIs and are found in both endosomes and lysosomes of macrophages have been shown to play an important role in macrophage-mediated antigen presentation and hence in cellular immune responses.[29–32] Selective inhibition of cysteine proteinase activity has been shown to profoundly alter the antigen-presenting capacity of macrophages.[33] Observations such as these suggest that by virtue of inhibiting cysteine proteinase catalyzed proteolytic degradation, stefins and other CPIs may influence the intracellular processes involved in antigen presentation and thereby modulate the interactions between antigen-presenting macrophages and T cells required for normal immune responses. By extension, it is possible that the increases in stefin protein levels detected in various tissues of motheaten mice might be relevant to the immune deficiency manifested by these animals. However, the precise mechanisms whereby increased stefin expression contributes to pathogenesis in these mutant mice is unclear, particularly because the increased levels of these proteins largely reflect expansion in the myelomonocytic population and are therefore probably associated with concomitant increases in levels of various cysteine proteinases.

In addition to their role in inflammation, both cysteine proteinases and their inhibitors have been implicated in cell differentiation and transformation.[34–41] For example, although stefin expression has been detected in all layers of the normal epidermis, levels of stefin synthesis appear to vary inversely with stage of differentiation.[34–36] These and other data concerning stefin expression in the skin suggest that these proteins are involved in the regulation of epidermal differentiation. Synthetic CPIs have also been shown to induce the transformation of NIH-3T3 cells in culture[38] and changes in the subcellular distribution of both cysteine proteinases and their inhibitors have been described in various types of tumors.[39–41] These data suggest that stefins contribute in some manner to malignant transformation, a possibility consistent with our recent finding that mice heterozygous for the *me*^{v} and *me* mutations are unusually prone to developing lymphomas and adenocarcinomas, respectively.[42] Conversely, reduction in CPI activities may also be relevant to oncogenesis, as suggested by the finding of reduced total CPI inhibitory activity in conjunction with increased cathepsin B and L activities in breast cancer tissues.[43] Therefore, although the precise manner wherein CPIs might influence cellular transformation remains to be determined the available data suggest that CPI activity is relevant to tumor development.

In summary, the data presented here confirm the aberrant expression of stefin A proteins in motheaten mice and strongly suggest that this abnormality reflects the overexpansion of stefin-producing myelomonocytic cells in these mice. The *me* phenotype is now known to reflect loss of function mutations in the gene encoding PTP1C[11,12] and thus the massive expansion of myelomonocytic cells observed in these mice implies an important role for PTP1C in down-regulating the differentiation and/or proliferation of these cells. Whether the effects of impaired PTP1C function are more specifically manifested in a stefin-expressing subpopulation of myelomonocytic cells remains to be determined. However, the data presented here provide a framework for further investigations into the functional relevance of stefin A proteins to both normal cellular physiology and to the immunopathogenesis of *me* and other chronic inflammatory disorders.

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